

Polypyrrole Based Nano-Biosensor for Sensing Low Concentrations of Hydrogen Peroxide

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Abstract—This paper discuss about a novel technique of developing a nano-structured polypyrrole biosensor for measurement of very low concentrations of hydrogen peroxide in liquid media. The proposed fabrication method is very effective in growing a nano-structured conducting polymer layer on a planar conducting substrate. In addition enzyme loading was done under a high electric field of 1000V/m. The developed sensor provides a liner range of 0-200 μ M of hydrogen peroxide and a measurement sensitivity of 6.5Acm⁻²M⁻¹.

Keywords—polypyrrole, biosensor, amperometric, Hydrogen peroxide, pulsed polymerization

I. INTRODUCTION

Hydrogen peroxide(H₂O₂) is a commonly used chemical in industries such as pharmaceutical, food, plastic processing, cosmetics, and cleaning. Higher concentrations of H₂O₂ can cause serious damage to skin if direct contact has been made[1-3]. It is highly possible that hydrogen peroxide being released to the natural environment through these industrial processes. Even low concentrations of H₂O₂ can change the ecosystem balance beyond acceptable limits in the long term if it is added to natural water streams. H₂O₂ is not only has its presence in industrial waste, but also in many biological processes such as highly selective enzyme catalytic reactions. The concentrations in these biological processes are very small and therefore it is very important to measure H₂O₂ in low concentrations accurately.

H₂O₂ sensors mainly fall into two categories as enzymatic and non-enzymatic sensors. Enzyme based hydrogen peroxide sensors are the most common in literature[4-6], but there are non-enzyme based H₂O₂ sensors and they are also becoming popular now[7-9]. The enzyme based biosensor performance dependent on the electrical connectivity between the enzyme and the sensor substrate material. There are many literatures on introduction of different interface materials between these two including carbon nanotubes, metal nano-particles and conducting polymers etc. Research has been done on enhancing the biocompatibility of these interfacing materials to keep the enzyme activity for a long time, especially employing lipids and biopolymer chitosan have been investigated as successful candidates[10,11].

Horseradish peroxidase(HRP) has the highest demand among all enzymes investigated for hydrogen peroxide measurements. Therefore it has been chosen as the enzyme in

the developed nano-biosensor. One of the greatest challenges is to retain a higher percentage of enzyme on the sensor electrode surface and providing a fast electron transfer during the reaction. We have chosen a conducting polymer layer as our interfacing media for this purpose. polyaniline and polypyrrole are the most commonly used conducting polymers in biosensors for their inherent stability in atmospheric conditions and biocompatibility. In this research polypyrrole has been chosen due to authors' excessive experience in developing nano-biosensors using polypyrrole.

There are different enzyme loading technologies including physical adsorption, chemical bonding, copolymerization, cross linking etc.[12]. Each of these techniques has its own advantages and disadvantages. For example, a technique provides high sensitivity may suffer from slower response time. In this paper it presents a novel method of enzyme loading by applying a high electric field to the electrode during adsorption process. This effect in conjunction with the nano-structured surface morphology caused by pulsed electro-deposition of polypyrrole resulted in a high performance hydrogen peroxide sensor. This method is a very low-cost and simple fabrication process in comparison to most of the other expensive and complex methods to fabricate a conducting nano-structure suitable substrate to immobilize HRP.

II. EXPERIMENTAL

A. Material and apparatus

All the chemicals used were of analytical grade from Sigma Aldrich or Wako chemicals, Japan. Horseradish peroxidase (EC 1.11.1.7, 225 U/mg), pyrrole monomer, sodium hexafluorophosphate(NaPF₆) and Hydrogen Peroxide(30%) were purchased from Wako, Japan. Pyrrole monomer was distilled before use. Phosphate buffer solution (PBS) was freshly prepared daily for experiments by using KH₂PO₄ and Na₂HPO₄ and the pH was adjusted to 6.50 by using a Cyberscan pH 100/RS232 portable pH meter and kept at room temperature for settling.

Sensor electrode substrates were prepared using Indium Tin Oxide(ITO) planar electrodes. These were selected as they have very good conductivity and extremely planar surface morphology where the effect of pulsed deposition can clearly be seen in comparison to electrodes developed with a constant potential. Platinum sputtering was done by JEOL quick coater JFC1500. All electrochemical experiments were carried out

with Hokuto Denko HSV-100 Automatic Polarization System. All these experiments were carried out at 20-25°C.

B. Sensor Fabrication

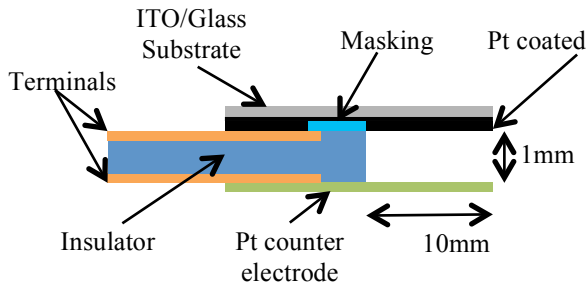


Fig.1. Schematic of the cross section of sensor fabrication assembly(not to the scale)

Initial part of the sensor fabrication process was very similar to the process described in [13-16]. First of all, ITO was prepared by cutting them into 10mm x 25mm rectangles and then they were sonicated in acetone for about 20 minutes and dried at 40°C. Then they were inspected under visual microscope for any unwanted residue deposits from the sonic cleaning process. Once passed the quality control, they were placed in DC Plasma sputtering machine (JFC1500) and a 50nm of a Pt layer was deposited under low pressure Argon(Ar) gas. Next the electrode substrates were masked by a thin layer of insulating ink to have a 10mm x 10mm window opened to polymerize sensor surface at one end and the other end is open for terminal connection.

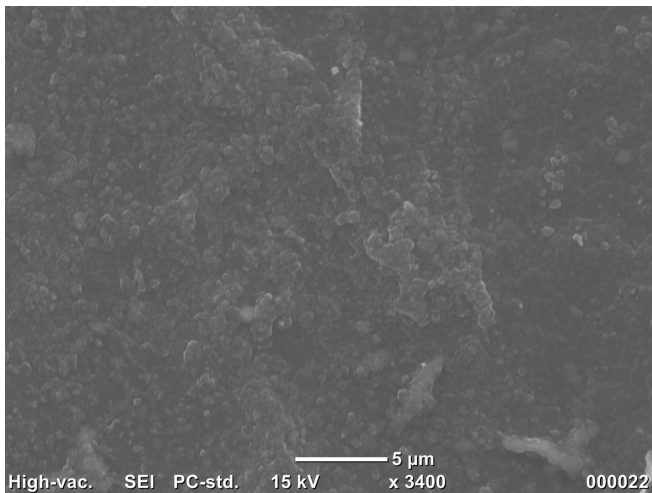


Fig.2. Surface morphology of the prepared polypyrrole film on Pt coated ITO

Pyrrole monomer was dissolved in distilled water to make a 0.05M solution and doped with 0.1M NaPF₆. The prepared substrate was placed in an arrangement as shown in Fig.1 and working electrodes of HSV-100 were connected to the terminal in contact with Pt modified ITO and reference and counter electrodes were connected to Pt counter electrode. An adequate amount of prepared pyrrole monomer was placed in between the ITO and Pt counter electrodes (10mm x 10mm x 1mm

cavity). Then a sequence of twenty, 1Volt square pulses of 0.5Hz were applied making it a 1000V/m electric field between the electrodes. These pulses electropolymerize the NaPF₆ doped pyrrole monomer to fabricate a nano-structured polypyrrole film on top of Pt modified ITO substrate. Then the monomer solution was removed and the sensor electrode assembly was washed gently in PBS solution and dried in air at 4°C.

Fig. 2 shows the surface morphology of the developed polypyrrole sensor where a large number of 100nm – 500nm nodules can be seen. The large islands are the places the film has overgrown. Most of the left part of the film shown in Fig.2 has a uniform distribution of nano-scale nodules. These nano-structured surface is the one which houses horseradish peroxidase during immobilizing process and subsequently act as the interfacing layer. It is really important to grow this to a size, which optimizes the morphology to match with the enzyme size. This was done through a trial and error process and it was found that 20 of 1V 0.5Hz gave the maximum performances.

C. Enzyme immobilizing

There are different techniques used in the field for immobilizing an enzyme onto a biosensor substrate. Physical adsorption, copolymerization, and cross linking are some of them[12]. However, in this research it has been employed a new method developed by the authors[15,16].

A stock solution of Horseradish Peroxidase(HRP) was prepared by mixing at a ratio of 1mg of HRP in 1ml of PBS(PH6.5) and kept at 4°C. 5μl of HRP from the stock solution is taken out of the refrigerator and kept outside to settle into room temperature. The sensor fabrication assembly is held to have the plates horizontally one above the other making it possible to inject the liquid in between them. The assembly terminals were again connected to the HSV-100 polarization system and the cavity is filled with PBS solution at room temperature. When filling the cavity it was taken care not to overfill it and leave some space for HRP. Then the 5μl of HRP is injected to the PHS solution in the cavity. Then a potential of 1V was applied across electrodes for twenty minutes and the solution was carefully removed from the cavity. The HRP loaded nano-biosensor is washed gently in PBS solution and allowed to dry at 4°C. Once air dried, the ITO/Pt/HRP nano-biosensor is ready for measurements.

D. Amperometric measurements

The prepared nano-biosensor assembly is immersed vertically in a 40ml of PBS solution where a micro magnetic bead rotating at non-turbulent low speed to mix the hydrogen peroxide homogeneously when added. It was taken care not to over spin the stirring magnetic bead as a turbulence flow would affect the reaction probability. A -0.1V potential was applied to the sensor against a Ag/AgCl reference electrode. After an adequate settling time, 25μM of H₂O₂ was added from a stock solution settled at the same temperature every 60 seconds to the system and the amperometric response was recorded. The response current is plotted online by the HSV-100 instrument and this process was continued until the amperometric reading become very noisy.

III. RESULTS AND DISCUSSION

A. Amperometric response

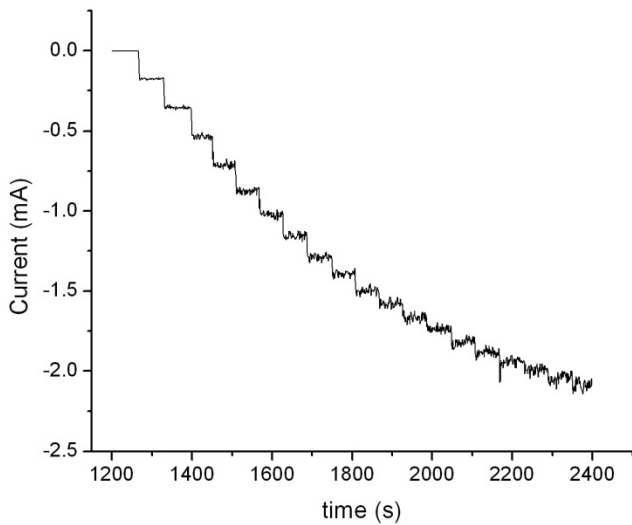


Fig. 3. Current measurement of the ITO/Pt/Ppy/HRP nano-biosensor with addition of H_2O_2 in steps of $0.25\mu\text{M}$ at 60 seconds intervals.

The response shown in Fig. 3 is for measurement of hydrogen peroxide in the testing media at an applied potential of -0.1V . Above graph shows the rapid increment in current for increments of $25\mu\text{M}$ at 60 seconds intervals. However, it can be seen from the Fig.3 that the signal to noise ratio becomes higher at higher concentrations and the response become less as the concentration increases. This determines the linear range of the sensor.

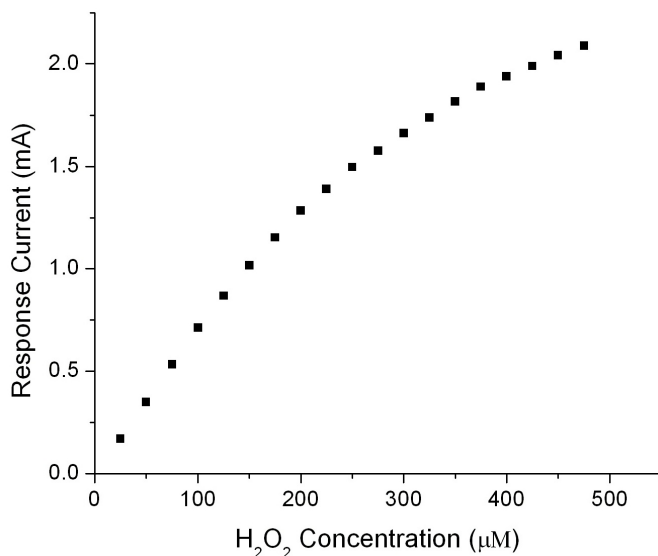


Fig. 4 Calibration curve for the ITO/Pt/PPy/HRP nano-biosensor

The calibration curve shown in Fig.4 was obtained from the raw data shown in Fig.3. It can be clearly seen from the above calibration curve that the developed sensor has a very

good linear range and sensitivity for hydrogen peroxide concentration determination. The linear range is from $0 - 200\mu\text{M}$ and the sensitivity was calculated to be $6.5\text{Acm}^{-2}\text{M}^{-1}$.

B. Discussion

The surface morphology of the polypyrrole film electrodeposited on the Pt modified ITO shown in Fig.2 clearly indicates that there are very high number of 100nm to 500nm nodules. Since the base substrate of ITO is one of the most planar conducting surfaces one can find, it is fair to assume that it is not changed much during the 50nm of Pt deposition by vacuum plasma process. The Pt here acts as a catalytic layer for electron transfer. The pulsed potential applied to the working electrode initializes the electro-polymerization process, but this activity is interrupted after one second as the applied potential is made zero. Then at the next pulse when the process restarts there is no guarantee that the polymerization restarts from the previously formed polymer dots. In contrast, they may start a new polymer dot forming for another one second. This process continues for twenty times and that is what creates a very high number of nano-scale nodules on the surface.

We have tried to load enzyme by physical adsorption by applying it for 30 minutes and tested that the sensor had very poor performances. In the new HRP deposition process the two parallel electrodes were separated only by 1mm perpendicular distance and applied 1V potential creates 1000V/m electric field. This high electric field assists HRP molecules to get bonded to the polypyrrole substrate properly. The increase in the response current proves that the amount of enzyme bonded is significantly increased through this process.

The lower end of the raw data in Fig. 3 illustrates that it has a very stable linear range of $0 - 200\mu\text{M}$. With the smooth curvature in the upper end suggests that a nonlinear second order polynomial relationship can easily be derived for this curve for the upper end and hence the full measurement range can be utilized with the help of modern digital signal processing techniques to calculate the hydrogen peroxide concentration measured in any given sample in the total range of $0 - 500\mu\text{M}$.

IV. CONCLUSION

The developed novel hydrogen peroxide sensor has comparatively a high sensitivity and a very good linear range for this class of a nano-biosensor. The proposed pulsed electro polymerization of polypyrrole provides a nano-structured substrate suitable for HRP loading. The HRP loading is further enhanced through application of high electric field. This combined process enables loading and retaining of relatively high number of enzyme molecules in the sensor substrate.

The results revealed that the sensor has a very high sensitivity and a good liner range , capable of detecting traces of hydrogen peroxide in very low (μM level) concentrations. This kind of a sensor can easily be integrated with low-cost front-end electronics to develop an affordable sensor to monitor environmental hydrogen peroxide levels.

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